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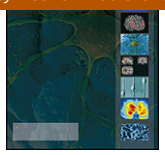

H-reflex down-conditioning greatly increases the number of identifiable GABAergic interneurons in rat ventral horn

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ABSTRACT

H-reflex down-conditioning increases GABAergic terminals on spinal cord motoneurons. To explore the origins of these terminals, we studied the numbers and distributions of spinal cord GABAergic interneurons. The number of identifiable GABAergic interneurons in the ventral horn was 78% greater in rats in which down-conditioning was successful than in naive rats or rats in which down-conditioning failed. No increase occurred in other spinal lamina or on the contralateral side. This finding supports the hypothesis that the corticospinal tract influence that induces the motoneuron plasticity underlying down-conditioning reaches the motoneuron through GABAergic interneurons in the ventral horn.

Operant conditioning of the H-reflex, the electrical analog of the spinal stretch reflex (SSR), is a simple model for exploring the acquisition and maintenance of motor skills, which can be defined as adaptive behavior acquired through practice [51]. Because the reflex is influenced by descending input from the brain, its pathway can be operantly conditioned. Motivated by an operant conditioning paradigm in which reward depends on reflex size, monkeys [49,53], humans [20,42], rats [9], and mice [8] can gradually increase or decrease the H-reflex or the SSR. These changes, i.e., a smaller or larger H-reflex, constitute simple motor skills. The acquisition of these skills is associated with plasticity in the spinal cord and the brain [6,7,12,14,16,17,21,36,47,54,50] ([51] for review). This acquisition requires the corticospinal tract (CST), but not the other major descending tracts [13,15,10,11].

Monkey and rat data indicate that down-conditioning of the H-reflex is due largely to a positive shift in the firing threshold of the spinal motoneurons that produce the reflex [6]. Since the CST does not contact these motoneurons directly [2,24,32,56], the CST influence responsible for the threshold change is presumably conveyed via spinal interneurons. To identify these interneurons, we first studied the effects of conditioning on synaptic terminals on the motoneurons. We found that successful down-conditioning is associated with a marked increase in the number of identifiable GABAergic terminals on the motoneurons [48]. This increase does not occur in rats in which down-conditioning is not successful. We hypothesize that these GABAergic terminals act through metabotrophic GABA-B receptor to alter sodium channels in the motoneuron membrane, and thereby change firing threshold [7,24].

GABAergic terminals on motoneurons derive from the ventromedullary reticular formation via the ipsilateral dorsolateral funiculus [25]. In addition, interneurons in spinal laminae VI–IX send motoneurons inhibitory projections that are largely GABAergic and/or glycinergic [1,3,5,19,22,26,27,30,31,38]. However, transection of the entire ipsilateral lateral column, including the dorsolateral funiculus, does not impair conditioning [13,11]. Thus, if these terminals are responsible for conditioning, they probably derive from interneurons in spinal laminae VI–IX. Interneurons in these laminae are contacted by CST axons [28,33,45].

To evaluate the hypothesis that GABAergic interneurons in laminae VI–IX are the origin of the GABAergic terminals changed by down-conditioning, we analyzed GABAergic interneurons in lumbar spinal cords of successful and unsuccessful down-conditioned rats and naive rats. The number of identifiable GABAergic interneurons in laminae VII and IX was markedly increased in successful rats only. These results support the hypothesis that the GABAergic interneurons are the CST-motoneuron link responsible for the positive shift in motoneuron firing threshold that underlies the H-reflex decrease.

Subjects were 16 young adult (about 5 months old) male Sprague–Dawley rats weighing 497 (±21SD) g at the time of euthanasia and perfusion. Ten had undergone electrode implan-

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tation and H-reflex down-conditioning. The other six constituted a naive control group. All were studied anatomically as described here. All procedures satisfied the "*Guide for the Care and Use of Laboratory Animals*" of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, D.C., 1996) and had been reviewed and approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. The protocols for H-reflex conditioning, motoneuron labeling, and immunohistochemical processing are fully described elsewhere [12,48,52] and summarized here. Other procedures are described in detail.

Under general anesthesia [pentobarbital sodium (65 mg/kg), i.p.], each of 10 rats was implanted with stimulating and recording electrodes. To elicit the H-reflex, a nerve-stimulating cuff was placed on the right posterior tibial nerve just proximal to the triceps surae branches. To record soleus EMG activity, fine-wire electrodes were inserted in the right soleus muscle. The Teflon-coated wires from the nerve cuff and the EMG recording electrodes passed subcutaneously to a connector mounted on the skull.

Data collection started at least 20 days after implantation. During data collection, each rat lived in a standard rat cage with a flexible cable attached to the head plug. The cable, which allowed the rat to move freely about the cage, carried the wires from the electrodes to an electronic swivel above the cage, from which they passed to an EMG amplifier and a nerve-cuff stimulation unit. The rat had free access to water and food, except that, during H-reflex down-conditioning, it received food mainly by performing the task described below. Animal well-being was carefully checked several times each day, and body weight was measured weekly. Laboratory lighting was reduced from 21:00 to 06:00 h each day.

A computer system continuously (24 h/day, 7 day/wk) monitored soleus EMG and controlled the nerve-cuff stimulus. Whenever the absolute value (i.e., equivalent to the full-wave rectified value) of background (i.e., ongoing) EMG stayed within a defined range for a randomly varying 2.3–2.7 s period, a stimulus pulse (typically 0.5 ms long) was delivered by the nerve cuff. Pulse amplitude was initially set to produce a small M response (i.e., it was set just above M response threshold), and then was automatically adjusted after each trial to maintain EMG amplitude for the M response interval (typically 2.0–4.5 ms after stimulation) unchanged. Thus, the background EMG (reflecting soleus motoneuron tone at the time of H-reflex elicitation) and the M-response (reflecting the effective strength of the nerve-cuff stimulus) remained stable throughout data collection.

Under the control mode, the computer simply measured the absolute value of soleus EMG for 50 ms following the stimulus. Under the down-conditioning mode, it gave a reward (i.e., a food pellet) 200 ms after stimulation if EMG amplitude in the H-reflex interval (i.e., typically 6.0–10.0 ms after stimulation) was below a criterion value. In the course of its daily activity, the animal usually satisfied the background EMG requirement, and thus received nerve-cuff stimulation 2700–7800 times per day. H-reflex size was calculated as average EMG amplitude in the H-reflex interval minus average background EMG amplitude, and was expressed in units of average background EMG amplitude. Each rat was first exposed to the control mode for 20 days to determine the control H-reflex size and was then exposed to the down-conditioning mode for 50 days. Finally the rat was euthanized and perfused as described below.

To determine the final effect of the down-conditioning mode on H-reflex size, average H-reflex size for the final 10 days of the 50-day exposure was calculated as per cent of the control H-reflex size (i.e., the average of the final 10 control-mode days). Down-conditioning was considered to be successful if final H-reflex size was ≤80% of control H-reflex size [9,55].

H-reflex conditioning was successful in 6 of the 10 down-conditioned rats. (This success rate was not significantly different from the rate of 75% found in all 72 rats down-conditioned to date [9–11,13–15].) In the six successful down-conditioned rats (i.e., DS rats), final H-reflex size averaged 51 (±3SE)% of its control value. In each of the four unsuccessful rats (i.e., DF (i.e., failed) rats), final H-reflex size was within 20% of its control value, and the average value for the four rats was 105 (±7SE)% of control. For all 10 rats, background EMG and M-response remained stable throughout data collection.

Each rat was euthanized by an overdose of sodium pentobarbital and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The lumbosacral spinal cord was removed and postfixed for 2 h, washed with 0.05 M phosphate buffer containing 137 mM NaCl (PBS, pH 7.4), and infiltrated with 30% sucrose for 24 h. The spinal cord containing the soleus motoneuron pool (i.e., L4-6) was blocked, embedded in OCT compound (Tissue-Tek), and frozen on dry ice. Transverse 25-μm frozen sections were cut with a cryostat, mounted onto precoated glass slides (Superfrost; Fisher). The slides were then stored in a low-temperature freezer (−80 °C) before further immunohistochemistry processing.

The standard avidin–biotin complex (ABC)-peroxidase system (ABC Elite; Vector Laboratories, Burlingame, CA) was used to assess GAD67-immunoreactivity as described previously [48]. GAD67-immunoreactivity, which is a standard method for labeling GABAergic terminals has also been found useful for labeling GABAergic neurons in adult as well as young animals [3,4,18,30,37,39,43,44,46]. All processing was conducted at room temperature (20 °C). Every other 25-μm section through the spinal cord containing the soleus motoneurons was washed three times (10 min each) with 0.05 M phosphate-buffered saline containing 0.1% Triton X-100 (PBST, pH 7.4), blocked with 5%-normal goat serum, and incubated with rabbit anti-GAD67 polyclonal antibody (K2 antibody (Chemicon, Temecula, CA) at 1:2000 dilution) in PBST containing 3% bovine serum albumin for 18–20 h [29]. The secondary goat anti-rabbit biotinylated antibody (1:200 in PBS) was applied for 1.5 h. Endogenous peroxidase activity was quenched by 0.3% H_2O_2 for 30 min, and the sections were reacted with the avidin–biotin complex for 1.5 h (1:100 in PBS). They were washed in 0.05 M Tris–HCl buffer (TBS, pH adjusted to 7.6 before color development). The sections were reacted with 0.04% DAB (Sigma, St. Louis) solution and 0.006% H_2O_2 for 8 min to optimize the signal-to-noise ratio. Finally, they were washed for 40 min, dehydrated, and mounted with Permount. Each processing session included matching sections from all three rat groups.

Every other GAD67-labeled section was examined at low magnification (i.e., 50×, with a 4× objective) and then examined and photographed at high magnification (500×, with a 40× objective) with an Olympus BH2-RFCA brightfield microscope equipped with an Olympus DP70 digital camera at fixed illumination. These high-magnification images were coded for blinded analysis using the image J program (NIH, version 1.29x) by two people (YW and SP) who worked independently and then resolved any differences by discussion. Analysis was confined to those GAD67-positive neurons in the ventral horns of GAD67-labeled sections that had: a clearly defined somatic border; a nucleus and/or at least one dendritic process; an average soma diameter (average of the long and short somatic axes) under 25 μm; and an average somatic area under 400 μm^2. Neurons that satisfied all these criteria were identified as GABAergic interneurons, and each was assigned a laminar location according to Molander et al. [35]. For each GABAergic interneuron, we determined soma diameter (i.e., Feret's diameter, equivalent to the long axis of the soma), soma area, and the average luminance of the entire soma (which reflected the intensity of GAD67-immunoreactivity (GAD-IR) [48]).

We compared the GAD67-labeled interneuron data from three experimental groups: rats in which H-reflex down-conditioning

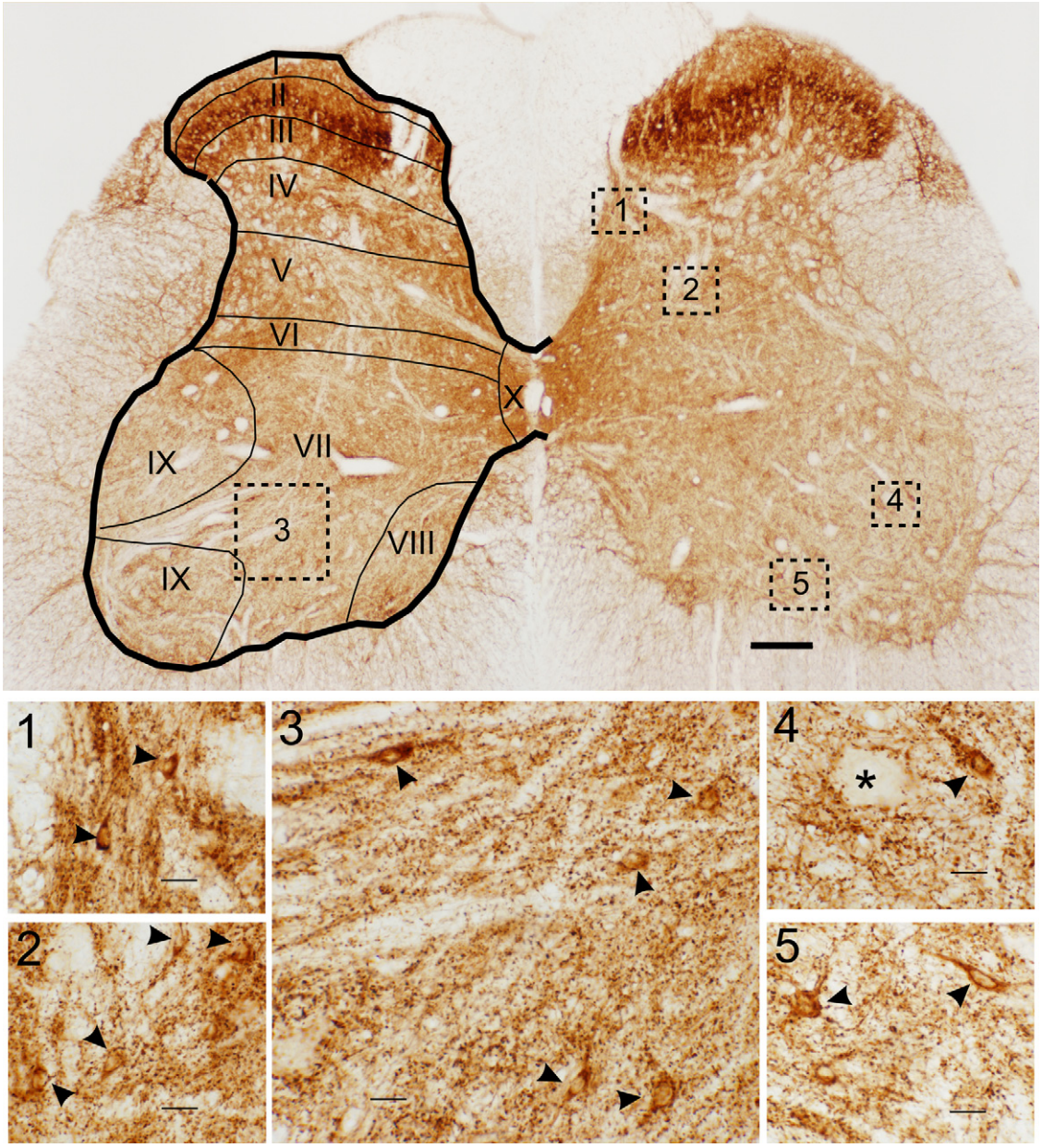

Fig. 1. Low (top) and high magnification (bottom) photomicrographs showing location and size of glutamic acid decarboxylase-67 containing interneurons (GAD-INs) in a 25-μm section from the lumbar spinal cord of a naive control rat. Arrow heads in the high magnification photomicrographs indicate identifiable GAD-INs. The asterix in box 4 indicates a motoneuron. The GAD-INs in the lamina VII are larger than those in other areas. Most identifiable GAD-INs in the ventral horn appear to be contacted by GAD67-containing terminals. Scale bar is 200 μm for the top photomicrograph and 20 μm for the bottom ones.

was successful (DS rats), rats in which down-conditioning failed (DF rats), and naive control rats (NC rats). For each measure, the three groups were compared by one-way ANOVA. When a difference was detected with $P < 0.01$, it was then confirmed by nested ANOVA, with cells nested in rats, and rats nested in groups. Significant ($P < 0.01$) differences between specific groups were detected by the least squares means contrast value test.

Fig. 1 shows a spinal cord cross-section with lamina marked and examples of GABAergic interneurons from several locations. Putative GAD67-labeled interneurons (GAD-INs) were observed throughout the spinal cord gray matter. Their sizes, shapes, and distribution were consistent with Barber et al. [4]. In the superficial dorsal horn they were small (66 (±41SD) μm^2) and densely packed, and appeared as a dark brown cell layer (Fig. 1 (top)). In contrast, in the ventral horn they were larger (96 (±53SD) μm^2) and scattered (Fig. 1, boxes 3–5). Some were within the motoneuron pool (lamina IX) (Fig. 1, box 4).

The principal positive finding was that the number of GABAergic interneurons in the ventral horn laminae VII and IX was much greater (i.e., 78% greater) in rats with successful down-conditioning (DS) than in down-conditioning failed (DF) or naive control (NC) rats ($P < 0.0001$ DS vs DF and NC rats, respectively) (Fig. 2 and Table 1). There were no significant differences detected among the three groups for laminae VI and VIII ($P > 0.05$ for all comparisons). Down-conditioning induced increase in GAD-INs in DS rats occurred only on the same (i.e., the conditioned) side of the spinal cord, but not on the contralateral (i.e., the un-conditioned) side of the spinal cord. In the DS rats, the number of GAD-INs on the conditioned side of the spinal cord was significantly greater than that on the un-conditioned side ($P < 0.01$). There were no significant differ-

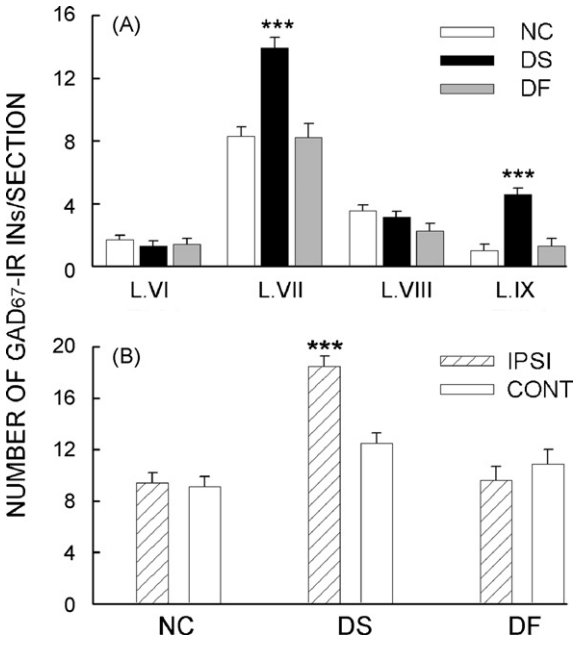

Fig. 2. (A) Average (±SEM) numbers of identifiable GABAergic interneurons in laminae VI, VII, VIII and IX per 25-μm section of lumbar spinal cord in successful down-conditioned (DS, black bars), unsuccessful (i.e., failed) down-conditioned (DF, gray bars), and naive control (NC, empty bars) rats. $^{***}P < 0.0001$ vs either NC rats or DF rats. (B) Average (±SEM) numbers of GABAergic interneurons per section in spinal laminae VII and IX of the ipsilateral (IPSI, i.e., conditioned side) and contralateral (CONT, i.e., unconditioned side) of the spinal cord for NC, DS, and DF rats. $^{***}P < 0.0001$ vs the contralateral spinal cord.

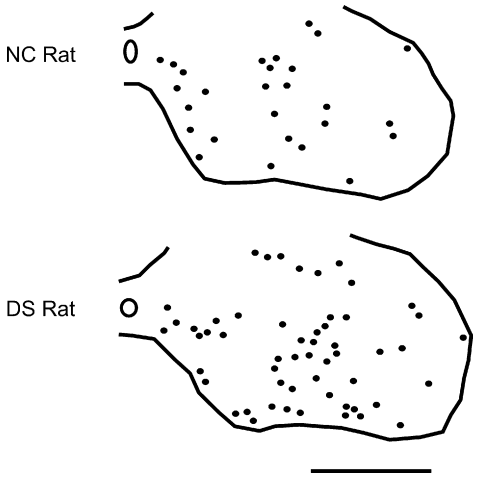

Fig. 3. Camera lucida drawings of ventral horn from L4-5 spinal cord sections from a naive rat (NC) (top) and a successfully down-conditioned rat (DS) (bottom) showing GAD67-positive interneurons (GAD-INs). Each drawing displays GAD-INs from two adjacent 25-μm sections. The number of GAD-INs is clearly greater in the DS rat than in the NC rat. Scale bar: 500 μm.

ences detected in DF and NC rats between the conditioned side and the un-conditioned side of the spinal cord. These very clear results concerning numbers of GABAergic interneurons are summarized in Fig. 2.

In contrast to the clear effect of successful down-conditioning on the number of GABAergic interneurons, there were no significant differences detected among the three groups in neuron diameter, area, or density of GAD-IR labeling (Table 1).

Fig. 3 shows camera lucida drawings of spinal cord intermediate and ventral horn from an NC rat and a DS rat with laminae VI–IX and the locations of GABAergic interneurons marked. The greater number of GABAergic interneurons in laminae VII and IX of the DS rat are clear, and contrasts with the lack of difference between the two rats for other lamina.

H-reflex down-conditioning requires the CST (but not other descending pathways) and is explained largely by a positive shift in motoneuron firing threshold [6,10,11]. However, the rat CST does not contact lumbar motoneurons [2,23,32,56]. Thus, the critical CST influence presumably reaches the motoneuron through spinal interneurons.

A recent study showed that successful (but not unsuccessful) down-conditioning greatly increases the number of identifiable GABAergic terminals on the motoneuron soma [48]. These terminals might act on metabotrophic receptors to induce the threshold shift underlying down-conditioning. Inhibitory terminals on the motoneuron derive mainly from interneurons spinal laminae VI–IX [5,19,22,26,27,30,38]. In addition, GABAergic terminals from the ventromedullary reticular formation project to motoneurons via the ipsilateral dorsolateral funiculus [25]. However, transection of the entire lateral column, including the dorsolateral funiculus, does not impair down-conditioning. Furthermore, the CST does project to interneurons in laminae VII–IX [28,33,45]. Thus, the present study addressed the hypothesis that spinal interneurons are the source of the modified GABAergic inputs to the motoneurons.

Our results support this hypothesis. The observations that successful down-conditioning greatly increases identifiable GABAergic interneurons in laminae VII and IX, that unsuccessful down-conditioning has no effect, and that no changes occur in the contralateral spinal cord suggest that these interneurons are the source of increased motoneuron terminals and that they have a key role in producing the reflex change.

In DS rats, the markedly increased numbers of identifiable GABAergic interneurons in the ventral horn and GABAergic terminals on the motoneuron may reflect increases in the proportions of interneurons and terminals expressing GAD67, rather than generation of new interneurons or terminals. Such changes in expression have been reported in other situations (e.g., [34,40,41]). It should

Table 1
Numbers of rats, numbers of ipsilateral (I) (i.e., conditioned) and contralateral (C) (i.e., unconditioned) sections, average (±SEM) numbers of GABAergic interneurons (GAD-INs) per section, and average (±SEM) somatic area, diameter, and GAD-IR labeling density for each GAD-IN from laminae VII and IX, for NC, DS, and DF rat groups. The number of GAD-INs per ipsilateral section was significantly greater in DS rats than in NC or DF rats ($^{*}P < 0.0001$ by one-way nested ANOVA). Furthermore, in DS rats, the number of GAD-INs per section was significantly greater on the ipsilateral side than on the contralateral side ($P < 0.0001$ by nested ANOVA). No significant differences were detected among the three rat groups or between the ipsilateral and contralateral sections of each group for average (±SEM) somatic area, diameter, or GAD-IR labeling density ($P > 0.05$ by one-way nested ANOVA).

Group	Rats (#)	Sections (#)	GAD-INs (#/section)	Area (μm^2)	Diameter (μm)	GAD-IR Density (%)
NC	6	I: 50	9.6 ± 0.7	89.5 ± 2.4	15.2 ± 0.2	31.4 ± 0.4
		C: 50	9.3 ± 0.7	90.5 ± 2.4	15.0 ± 0.2	31.6 ± 0.4
DS	6	I: 49	18.6 ± 1.1*	93.5 ± 1.7	15.1 ± 0.2	32.4 ± 0.2
		C: 48	12.7 ± 1.1	97.9 ± 1.2	15.6 ± 0.2	32.3 ± 0.3
DF	4	I: 30	10.1 ± 0.9	92.3 ± 3.1	15.7 ± 0.3	31.6 ± 0.5
		C: 28	10.5 ± 0.8	92.6 ± 2.9	15.4 ± 0.3	32.6 ± 0.4

also be noted that recent data suggest that decreased expression of GABA-B receptors on motoneurons plays a role in H-reflex up-conditioning [47].

The necessary next step in establishing the role of these interneurons in H-reflex down-conditioning is to confirm that they are contacted by the CST and do project to the motoneurons. At this point, we have established that the acquisition of a simple motor skill is associated with a striking change in the neurotransmitter content of a distinct population of spinal interneurons.

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